Further Insights into the Oxidation Chemistry and Biochemistry of the Serotonergic Neurotoxin 5,6-Dihydroxytryptamine

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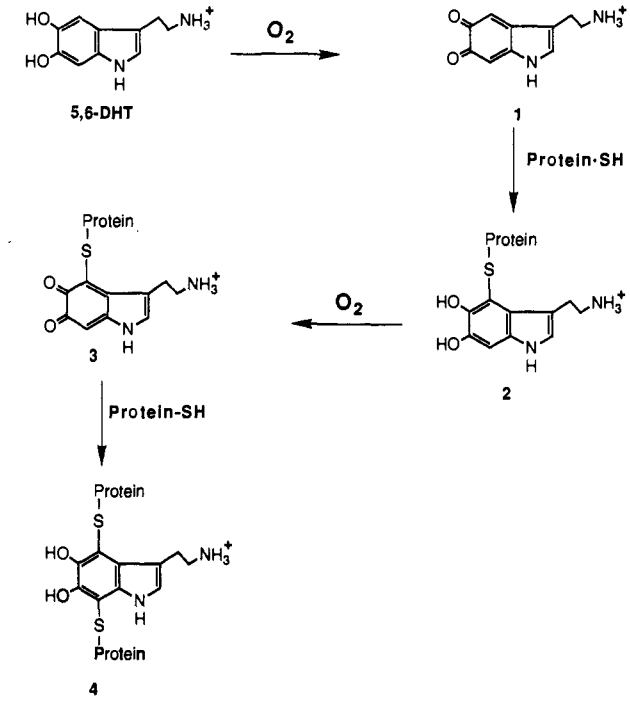
The neurodegenerative properties of the serotonergic neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) are widely believed to result from its autoxidation in the central nervous system. The autoxidation chemistry of 5,6-DHT has been studied in aqueous solution at pH 7.2. The reaction is initiated by direct oxidation of the indolamine by molecular oxygen with resultant formation of the corresponding o-quinone 1 and H_2O_2. A rapid nucleophilic attack by 5,6-DHT on 1 leads to 2,7'-bis(5,6-dihydroxytryptamine) (**6**) which is more rapidly autoxidized than 5,6-DHT to give the corresponding diquinone **7** along with 2 mol of H_2O_2. The accumulation of **6** in the reaction solution during the autoxidation of 5,6-DHT despite its more rapid autoxidation indicates that diquinone **7** chemically oxidizes 5,6-DHT (2 mol) to quinone 1 so that an autocatalytic cycle is established. The H_2O_2 formed as a byproduct of these autoxidation reactions can undergo Fenton chemistry catalyzed by trace transition metal ion contaminants with resultant formation of the hydroxyl radical, HO•, which directly oxidizes 5,6-DHT to a radical intermediate (**9a**/**9b**). This radical is directly attacked by O_2 to yield quinone 1 and superoxide radical anion, $O_2^{\bullet-}$, which further facilitates Fenton chemistry by reducing, inter alia, Fe^{3+} to Fe^{2+}. A minor side reaction of 1 with water leads to formation of at least two trihydroxytryptamines. Diquinone **7** ultimately reacts with **6**, 5,6-DHT, and perhaps trihydroxytryptamines, leading via a sequence of coupling and oxidation reactions to a black indolic melanin polymer. Enzymes such as tyrosinase, ceruloplasmin, and peroxidase and rat brain mitochondria catalyze the oxidation of 5,6-DHT to form dimer **7** and, ultimately, indolic melanin. The role of the autoxidation and the enzyme-mediated and mitochondria-promoted oxidations of 5,6-DHT in expressing the neurodegenerative properties of the indolamine are discussed.

5,6-Dihydroxytryptamine (5,6-DHT) is a pharmacological tool used for selective chemical lesioning of serotonergic neurons.[1] The selectivity of 5,6-DHT almost certainly derives from its high-affinity uptake by the serotonergic membrane pump. However, the molecular mechanisms by which 5,6-DHT expresses its neurodegenerative properties remain in question. It is rather widely believed that the neurotoxicity of 5,6-DHT stems from an inherent chemical property, namely, ease of autoxidation (i.e., oxidation by dissolved oxygen at physiological pH without catalysis by an enzyme). One principal theory has been advanced which relates the autoxidation of 5,6-DHT to its neurodegenerative properties. This theory proposes that autoxidation of 5,6-DHT generates an electrophilic o-quinone (1) which alkylates and cross-links neuronal membrane proteins as conceptualized in Scheme I.[2] Experiments with radiolabeled 5,6-DHT provide some support for the suggestion that the autoxidation product(s) undergo covalent binding with protein nucleophiles both in vitro[2] and in vivo.[3] However, autoxidation of 5,6-DHT eventually leads to formation of an extremely insoluble, high molecular weight, melanin-like polymer. An unknown fraction of the radioactivity recovered with protein-bound radioactivity undoubtedly derives from this melanin. As a result the extent of covalent binding of the autoxidation products of 5,6-DHT cannot be accurately assessed based upon such radiolabeling studies. The nature of the products formed between putative electrophilic intermediates generated upon autoxidation of 5,6-DHT with protein nucleophiles or even with model peptides remains to be elucidated.

Klemm et al.[4] have reported that H_2O_2 is formed in near stoichiometric yield during the autoxidation of 5,6-DHT and it has been suggested that the autoxidation reaction

Scheme I



is autocatalytically promoted by H_2O_2. There does not appear to be any definitive evidence for formation of other reduced oxygen species (e.g., $O_2^{\bullet-}$, HO•) during the autoxidation reaction although superoxide dismutase apparently decreases the rate of the reaction.[4]

Cohen and Heikkila[5] first reported that rat brain mitochondria catalyze the formation of quinones from 5,6-DHT and proposed an interaction of the indolamine with electron-transport systems in mitochondria. Klemm et al.[4] have also noted that mitochondria promote the oxidation of 5,6-DHT by molecular oxygen. The observed increase in oxygen consumption was not influenced by monoamine oxidase-dependent deamination of the indolamine. The latter workers have postulated that 5,6-DHT can partic-

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ipate in the electron transfer of mitochondrial respiration beyond complex III.

In an attempt to probe the molecular mechanism by which 5,6-DHT expresses its neurodegenerative action, Sinhababu et al.[6] synthesized a series of analogues which were methylated at the 4- and/or 7-positions. This strategy was designed to block either simultaneously or independently the sites thought to be the loci of attack by protein nucleophiles on putative quinone intermediate 1 (see Scheme I). Substitution of methyl groups had no deleterious effects on the cytotoxicity of 5,6-DHT although some reduction in uptake affinity was noted compared to that of the unsubstituted compound. Furthermore, 4,7-dimethyl-5,6-dihydroxytryptamine was found to be at least 50 times more cytotoxic than 5,6-DHT. This observation is surprising because autoxidation of the latter compound should yield a quinone intermediate in which both of its electrophilic sites are blocked.

Numerous studies have demonstrated that 5,6-DHT has a profound effect on behavioral and physiological activity shortly after administration in vivo and before extensive neuronal degeneration can take place.[7] Recent studies indicate that these effects might be related to the fact that 5,6-DHT, or some rapidly formed product, initially acts to displace serotonin from vesicular stores into the cytoplasm where it can either be deaminated by monoamine oxidase or be released.[8]

The related serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) has long been thought to express its neurodegenerative effects by a similar mechanism to that of 5,6-DHT. Autoxidation of 5,7-DHT has been proposed to yield an electrophilic quinonimine which alkylates neuronal membranes[9] accompanied by formation of cytotoxic reduced oxygen species as byproducts. However, recent work has demonstrated that a quinonimine intermediate is probably not formed upon autoxidation of 5,7-DHT but rather that radical intermediates are formed.[6,10] Furthermore, the autoxidation products of 5,7-DHT have been isolated and structurally characterized[10] and preliminary results suggest that the neurodegenerative properties of the indolamine might be expressed by one of these products, 5-hydroxytryptamine-4,7-dione.[10,11]

Taken together, the above observations suggest that, in order to better understand the molecular mechanisms by which 5,6-DHT expresses its neurodegenerative properties, considerably more information about its oxidation chemistry and biochemistry is necessary. This information should include inter alia the mechanisms and products of the autoxidation reaction, the fate of molecular oxygen that is consumed in the reaction, the role of reactive reduced oxygen species that might be formed, and the catalytic effects of metal ions, mitochondria, and other enzyme systems endogenous to the central nervous system. As a first step to obtaining such information, we recently isolated the major autoxidation product of 5,6-DHT, 2,7'-bis(5,6-dihydroxytryptamine) (**6**).[12] Furthermore, by use

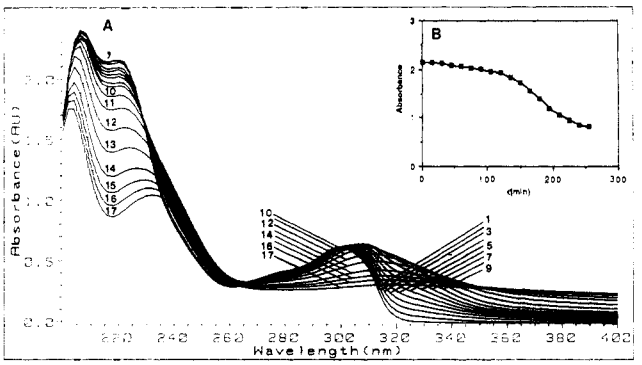

Figure 1. (A) Spectral changes during the autoxidation of 0.2 mM 5,6-dihydroxytryptamine in pH 7.2 phosphate buffer (μ = 0.1) at room temperature. Curve 1 is the spectrum of 5,6-DHT; curves 2–16 were recorded at 15-min intervals. (B) Variation of the absorbance at 218 nm during the autoxidation of 0.2 mM 5,6-DHT.

of high-performance liquid chromatography–mass spectrometry, two trihydroxytryptamines and a second dimer of 5,6-DHT were identified as minor autoxidation products. In this report chemical and biochemical approaches have been employed to gain further insights into the autoxidation of 5,6-DHT and to explore the catalytic role of various metal ions and enzyme systems in the oxidation chemistry of the neurotoxin.

Results

Spectral changes which accompany the autoxidation of 5,6-DHT in pH 7.2 phosphate buffer are shown in Figure 1A. The spectrum of 5,6-DHT (curve 1, Figure 1A exhibits bands at λ_{max} = 300, 280 (sh), and 218 nm. During the early stages of the autoxidation the band at 300 nm slowly shifts to longer wavelengths and grows; correspondingly, the band at 218 nm decreases. With a 0.2 mM solution of 5,6-DHT, the long-wavelength band reaches its maximal height at λ_{max} = 308 nm after approximately 120 min and then begins to decrease in height and shifts to even longer wavelengths. The band initially at 218 nm decreases only slightly during the initial 75–90 min of the autoxidation but then decreases more rapidly and shifts to longer wavelengths. The change in absorbance with time monitored at 218 nm (Figure 1B) demonstrates that after ca. 120 min the reaction accelerates. This acceleration in the latter stages of the reaction has been attributed to the oxidation of 5,6-DHT by H_2O_2 formed as a byproduct.[4] After approximately 4.5 h, autoxidation of 0.2–1 mM 5,6-DHT is complete. The remaining spectral band (λ_{max} 229 nm) is due to creatinine (Figure 1A). The general increase in absorbance which occurs throughout the entire UV–visible spectral region as the autoxidation reaction progresses is caused by the systematic formation of a black, polymeric, melanin-like precipitate in the solution.

An oxygen electrode tracing (Figure 2A) shows the rate of oxygen consumption from the incubation medium. With 1.0 mM 5,6-DHT (37 °C, pH 7.2 phosphate buffer) the initial rate of oxygen consumption is 8.6 ± 0.6 nmol of O_2/min. In pH 7.2 MOPS and pH 7.2 HEPES buffers the corresponding rates were 10.8 ± 0.5 and 8.0 ± 0.6 nmol of O_2/min, respectively, indicating that buffer constituents probably have only a minor effect on the oxygen consumption rate. However, increasing the ionic strength of the pH 7.2 phosphate buffer to 1.0 caused the initial oxygen consumption rate to increase to 13.9 ± 0.4 nmol of

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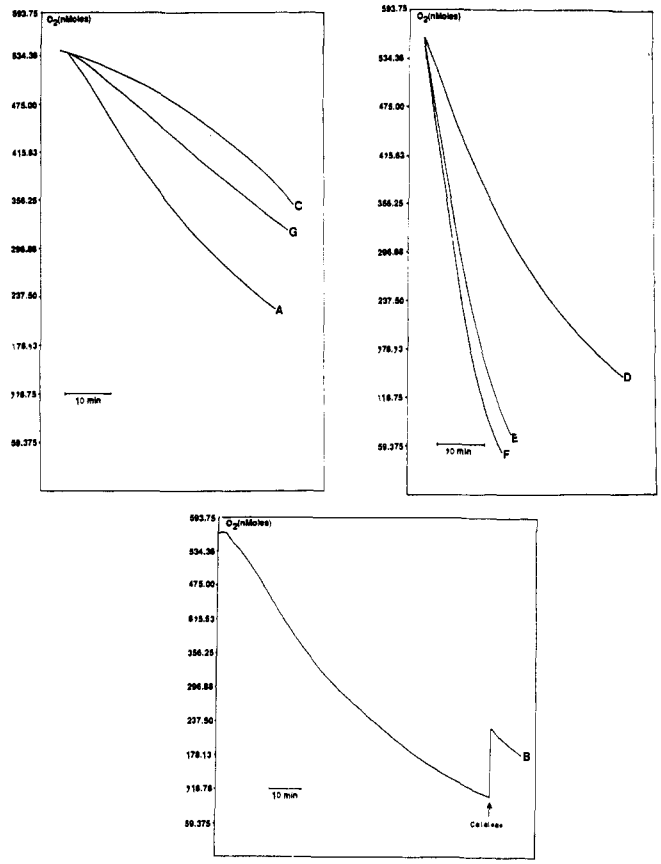

Figure 2. Oxygen consumption curves for (A) 1 mM 5,6-DHT (1.2 mg of 5,6-DHT in 3.0 mL of air-saturated phosphate buffer, pH 7.2; μ = 0.1) at 37 °C, (B) after 80 min of autoxidation 2000 units of catalase (1 mg, 10 μL) was added, (C) with added catalase (1 mg, 2000 units) in initial solution, (D) with added H_2O_2 (0.5 mM), (E) with added type VI peroxidase (3.0 units, 0.01 mg), (F) with added H_2O_2 (0.2 mM) and type VI peroxidase (3.0 units, 0.01 mg), (G) with added superoxide dismutase (1000 units). Traces were measured with an oxygen electrode assembly.

O_2/min. This effect is probably due to an increase in the concentration of trace transition metal contaminants present in the phosphate salts employed to prepare the buffer (see later discussion).

HPLC analysis of a partially autoxidized solution of 5,6-DHT shows several chromatographic peaks (Figure 3). HPLC peak 1 is due to creatinine (the creatinine sulfate salt of 5,6-DHT was used) and peak 6 to unreacted 5,6-DHT. The major product is clearly represented by HPLC peak 8 and is due to 2,7′-bis(5,6-dihydroxytryptamine) (**6**). The structure of **6** has been elucidated based upon high-resolution fast atom bombardment mass spectrometry and ^{1}H NMR spectroscopy (including 2D correlated spectroscopy and nuclear Overhauser experiments).[12] HPLC peak 2 is due to a trihydroxytryptamine and peak 7 to a second but incompletely characterized dimer of 5,6-DHT.[12] At later stages of the autoxidation reaction additional minor products appear but in view of their very low yields and instability these have not been fully characterized.

Formation of H_2O_2 during the Autoxidation Reaction. Several lines of evidence indicate that H_2O_2 is formed as a byproduct of the autoxidation of 5,6-DHT and that it plays a role in the overall autoxidation reaction. For example, addition of catalase to the reaction mixture after the autoxidation has proceeded for some time results in the liberation of molecular oxygen (Figure 2B). In such an experiment approximately 50% of the consumed O_2 can be accounted for as H_2O_2. The H_2O_2 generated in situ has an appreciable influence on the autoxidation reaction. For

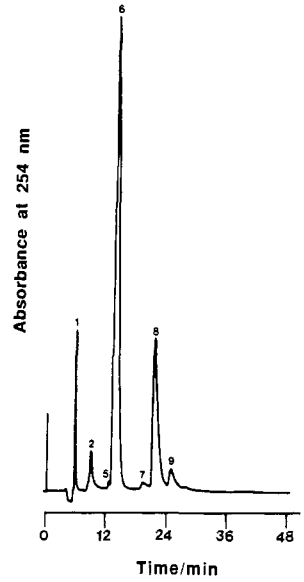

Figure 3. High-performance liquid chromatography of the product mixture formed after partial autoxidation of 2.0 mM 5,6-DHT in pH 7.2 phosphate buffer (μ = 0.1) for 150 min at room temperature. Chromatographic conditions are given in the Experimental Section.

example, when the autoxidation of 5,6-DHT (1 mM, pH 7.2 phosphate buffer, 37 °C) was carried out in the presence of catalase (1 mg, 2000 units) the initial rate of the reaction was decreased by more than 50% as measured with an oxygen electrode (3.4 ± 0.4 nmol of O_2/min) (Figure 2C). This decrease in oxygen consumption rate is not simply due to the return of O_2 to the solution as a result of the catalytic decomposition of H_2O_2. This is so because addition of H_2O_2 increased the rate of autoxidation of 5,6-DHT. For example the initial rate of oxygen consumption of 1 mM 5,6-DHT increased from 8.6 ± 0.6 to 18.2 ± 0.2 nmol of O_2/min when the initial reaction solution was made 0.5 mM in H_2O_2 (Figure 2D). HPLC analyses revealed that the product yields and distribution were not altered by addition of H_2O_2 to the reaction mixture. In the absence of molecular oxygen 5,6-DHT (0.2 mM) was only oxidized slowly in the presence of H_2O_2 (0.2 mM). Under the latter conditions, approximately 7% of the 5,6-DHT was oxidized in 4 h (compared to ≥90% in the normal autoxidation reaction). Addition of diethylenetriaminepentaacetic acid (DTPA, 1.0 mM) to the latter reaction mixture prevented any significant oxidation of 5,6-DHT. Accordingly, it may be concluded that H_2O_2 is not able to directly oxidize 5,6-DHT but that it requires the presence of trace concentrations of transition metal ions in order to contribute to the oxidation reaction. Furthermore, in the presence of O_2, the role of H_2O_2 in the overall autoxidation is further amplified.

Incubation of 5,6-DHT (3.0 mL, 1 mM in pH 7.2 phosphate buffer) with types VI, VIII, IX, and X peroxidase (3.0 units) and H_2O_2 (0.2 mM) resulted in the rapid oxidation of the indolamine and formation of a heavy, black precipitate within 30 min. HPLC analysis of the solution prior to the appearance of this precipitate revealed that **6** was the major product along with the other minor products characteristic of the autoxidation reaction. Thus, clearly, peroxidase catalyzes the oxidation of 5,6-DHT by H_2O_2. Accordingly, additional evidence for the generation of H_2O_2 in the autoxidation of 5,6-DHT was provided by including peroxidase in the reaction mixture. Type VI peroxidase (3.0 unit, 0.01 mg) included in a solution of 5,6-DHT (3.0 mL, 1 mM in pH 7.2 phosphate buffer, 37 °C) not only caused an acceleration of the oxidation as

Table I. Effect of Fe^{3+} and Cu^{2+} on the Initial Rate of Oxygen Consumption by 5,6-DHT[a]

metal ion or complexing agent	concn, μM	initial rate of oxygen consumption,[e] nmol of O_2/min
none	NA	8.6 ± 0.6
Fe^{3+} [b]	0.1	16.9 ± 0.1
	1.0	19.0 ± 0.1
	10.0	32.0 ± 0.1
Cu^{2+} [c]	0.1	22.9 ± 0.1
	1.0	25.4 ± 0.1
	10.0	72.7 ± 0.1
DTPA[d]	500	6.94 ± 0.2
	1000	3.16 ± 0.2

[a] 1.0 mM 5,6-DHT (3.0 mL) in air-saturated, pH 7.2 phosphate buffer, μ = 0.1 at 37 °C. [b] Added as $FeCl_3$. [c] Added as $CuSO_4$. [d] Diethylenetriaminepentaacetic acid. [e] Measured with an oxygen electrode assembly.

monitored by spectral changes but also caused an increase in the initial rate of oxygen consumption as measured with an oxygen electrode (Figure 2E). Thus, in the latter experiment the initial oxygen consumption rate was 32.6 ± 0.4 nmol of O_2/min (Figure 2E) compared to 8.6 ± 0.6 nmol of O_2/min in the absence of peroxidase (Figure 2A). When H_2O_2 (0.2 mM) was included in the latter reaction mixture the initial oxygen consumption rate increased further to 36.8 ± 0.5 nmol of O_2/min (Figure 2F).

Formation of Superoxide Radical Anion, $O_2^{\cdot-}$, in the Autoxidation Reaction. Superoxide dismutase (SOD) catalyzes the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2[13] and has been found to be a valuable tool to probe reactions which involve this radical.[14] SOD (1000 units, 50 μL) included in a solution of 5,6-DHT (3 mL, 1 mM in pH 7.2 phosphate buffer, 37 °C) decreased the initial O_2 consumption rate to 5.0 ± 0.5 nmol of O_2/min (Figure 2G) (compared to 8.6 ± 0.6 nmol of O_2/min in the absence of SOD). Increasing the amount of SOD to 2000 units (100 μL) caused only a minor additional decrease in the initial O_2 consumption rate to 4.60 ± 0.3 nmol of O_2/min. Denatured SOD (1000 units, 50 μL) caused a small enhancement in the rate of O_2 consumption by 5,6-DHT (10.1 ± 0.2 nmol O_2/min). These results suggested that $O_2^{\cdot-}$ is formed in the autoxidation of 5,6-DHT and that the radical plays some active role in the overall rate of the reaction. This was investigated by conducting the autoxidation in the presence of xanthine and xanthine oxidase. Xanthine oxidase generates $O_2^{\cdot-}$ when catalyzing the oxidation of xanthine to uric acid.[15-17] Incubation of 5,6-DHT (1.0 mM, 3.0 mL in pH 7.2 phosphate buffer, 37 °C) with xanthine oxidase (1.0 unit) caused a slight enhancement of the initial rate of oxygen consumption to 10.3 ± 0.2 nmol of O_2/min. The oxygen consumption rate increased to 12.9 ± 0.2 nmol of O_2/min when xanthine (0.01 mM) was included in the reaction mixture. Addition of SOD (1000 units, 50 μL) to the latter solution decreased the oxygen consumption rate to 10.16 ± 0.2 nmol of O_2/min. The oxygen consumption rate of 0.01 mM xanthine and xanthine oxidase (1.0 unit) was 2.74 nmol of O_2/min.

Table II. Effect of Rat Brain Mitochondria on the Rate of Oxygen Consumption by 5,6-DHT

wt of mitochondrial protein added, mg[a]	buffer system	initial rate of oxygen consumption,[c] mol of O_2/min
0	phosphate, pH 7.2[b]	8.61 ± 0.6
0	mannitol-tris, pH 7.4[c]	7.48 ± 0.5
0.2	mannitol-tris, pH 7.4	33.8 ± 0.2
0.4	mannitol-tris, pH 7.4	47.5 ± 0.2
0.8	mannitol-tris, pH 7.4	83.1 ± 0.2
0.8	phosphate, pH 7.2	72.8 ± 0.2

[a] Added to 1.0 mM 5,6-DHT in 3.0 mL of the appropriate buffer system at 37 °C. [b] Phosphate buffer, pH 7.2, μ = 0.1. [c] Measured with an oxygen electrode assembly at 37 °C.

Effects of Transition Metal Ions on the Autoxidation of 5,6-DHT. Both Fe^{3+} and Cu^{2+} greatly enhanced the rate of oxygen consumption by 5,6-DHT (Table I), an effect which became more pronounced with increasing concentrations of the metal ions. In the absence of oxygen, Fe^{3+} was unable to effect the oxidation of 5,6-DHT. However, under the same conditions Cu^{2+} rapidly oxidized 5,6-DHT. In the absence of added transition metal ions, DTPA, a strong chelating agent for transition metal ions (Fe^{3+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Mn^{3+}, Mn^{2+}),[18] caused a decrease in the rate of oxygen consumption by 5,6-DHT (Table I). Accordingly, it may be concluded that trace metal ions, which always contaminate the buffer salts employed, exert a catalytic effect on the autoxidation of 5,6-DHT. HPLC analyses of the product solutions formed as a result of the Fe^{3+}- and Cu^{2+}-catalyzed autoxidations of 5,6-DHT and by direct oxidation by Cu^{2+} in the absence of O_2 revealed that **6** was the major product.

Effects of Other Enzymes

The cuproenzyme tyrosinase greatly accelerated the oxidation of 5,6-DHT by molecular oxygen. Oxygen electrode measurements revealed that incubation of 1 mM 5,6-DHT (3.0 mL in pH 7.2 phosphate buffer, 37 °C) with tyrosinase (80 units, 0.033 mg) enhanced the initial rate of oxygen consumption to 18 ± 0.1 nmol of O_2/min. A 10-fold increase in the amount of tyrosinase added caused the initial rate of oxygen consumption to increase to 158 ± 0.2 nmol of O_2/min. Ceruloplasmin, another copper-containing protein,[19] also accelerated the oxidation of 5,6-DHT. Incubation of 1 mM 5,6-DHT (3.0 mL in pH 7.2 phosphate buffer, 37 °C) with ceruloplasmin (20.4 units) increased the initial oxygen consumption rate to 24.8 ± 0.1 nmol of O_2/min. With 204 units of ceruloplasmin this rate increased to 208 ± 0.2 nmol of O_2/min. HPLC analysis revealed that the tyrosinase- and ceruloplasmin-mediated oxidations of 5,6-DHT gave **6** as the major initial product.

Mitochrondria-Promoted Oxidation of 5,6-DHT. Freshly prepared rat brain mitochondria stimulated the initial rate of oxygen consumption, the rate increasing with increasing amounts of mitochondria (Table II). Similar enhancements have been noted previously.[4,5] However, the major product of the reaction has not previously been identified. With use of analytical and preparative HPLC methods, the major product of the mitochondria stimulated oxidation has been isolated and characterized, by using spectroscopic methods described elsewhere,[12] as **6**. Ultimately, the mitochondria-stimulated oxidation of 5,6-DHT results in formation of a black, insoluble polymer similar to that observed in the simple autoxidation reaction.

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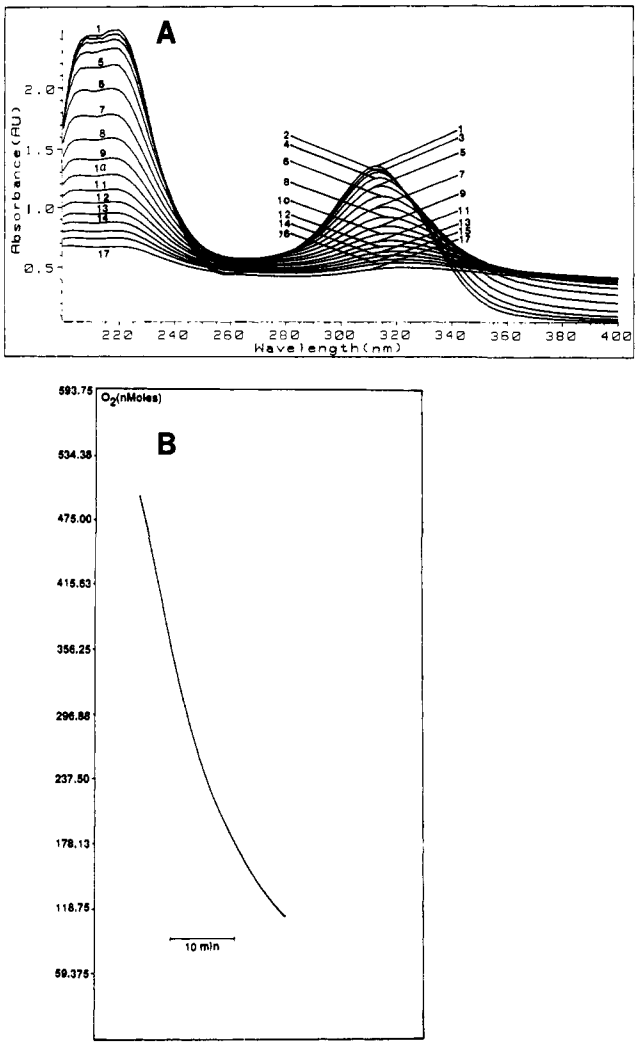


Figure 4. (A) Spectral changes during the autoxidation of 0.2 mM 2,7′-bis(5,6-dihydroxytryptamine) (**6**) in pH 7.2 phosphate buffer (μ = 0.1) at room temperature. Curve 1 is the spectrum of **6**; curves 2–17 were recorded at 2.5-min intervals. (B) Oxygen consumption curve for 0.2 mM **6** from air-saturated pH 7.2 phosphate buffer (μ = 0.1) at 37 °C measured with an oxygen electrode.

Autoxidation of 2,7′-Bis(5,6-dihydroxytryptamine) (6). Spectral changes which occur during the autoxidation of **6** at pH 7.2 are shown in Figure 4A. A black precipitate could be observed in the solution after 20 min. Using 0.2 mM **6**, complete autoxidation required <40 min (monitored by HPLC analysis) whereas at the same concentration level 5,6-DHT required ca. 4.5 h. HPLC analysis of the reaction solution throughout the autoxidation reaction showed a systematic decrease of the peak due to **6**, but no new chromatographic peaks appeared. Such behavior suggests that the primary autoxidation product(s) of **6** react very rapidly to form insoluble polymeric material(s). The initial oxygen consumption rate of 0.2 mM **6** (0.229 mg in 3.0 mL of pH 7.2 phosphate buffer, 37 °C) measured with an oxygen electrode was 26.7 ± 0.4 nmol of O_2/min (Figure 4B). At the same concentration the initial oxygen consumption rate for 5,6-DHT was 11.8 ± 0.4 nmol of O_2/min. Thus, both spectral and oxygen consumption rate data indicate that **6** is autoxidized at a significantly faster rate than 5,6-DHT.

Addition of catalase (1 mg, 2000 units) to the solution contained in the oxygen electrode assembly at a point when virtually all (≥90%) oxygen had been consumed by **6** (0.2 mM; 0.228 mg in 3.0 mL of pH 7.2 phosphate buffer) resulted in the liberation of 187.6 nmol of O_2. Thus, approximately 66% of the consumed oxygen is accounted for as H_2O_2. When catalase (1 mg, 2000 units) was added before initiating the autoxidation of **6** (0.2 mM, 3.0 mL) the initial oxygen consumption rate decreased to 21.0 ± 0.2 nmol of O_2/min. Conversely, addition of H_2O_2 (0.2 mM) to **6** (0.2 mM) caused the initial oxygen consumption rate to increase to 29.7 ± 0.3 nmol of O_2/min. In the absence of O_2, spectral studies revealed that **6** (0.2 mM) was oxidized only very slowly by H_2O_2 (0.2 mM). Thus, after 40 min less than 7% of the original **6** was oxidized. Under the same experimental conditions, except that DTPA (1.0 mM) was added, **6** was not oxidized. Superoxide dismutase (50 μL, 1000 units) decreased the rate of oxygen consumption of **6** (0.2 mM, 3.0 mL) to 19.0 ± 0.2 nmol of O_2/min. Xanthine oxidase (1 unit, 132 μL) slightly promoted the oxygen consumption rate of **6** (0.2 mM) to 28 ± 0.2 nmol of O_2/min. Addition of xanthine (0.0045 mg, 0.01 mM) to this reaction solution increased oxygen consumption to 30 ± 0.1 nmol of O_2/min. These results indicate that $O_2^{\cdot-}$ is generated in the autoxidation of **6** and that it plays an active role in the reaction.

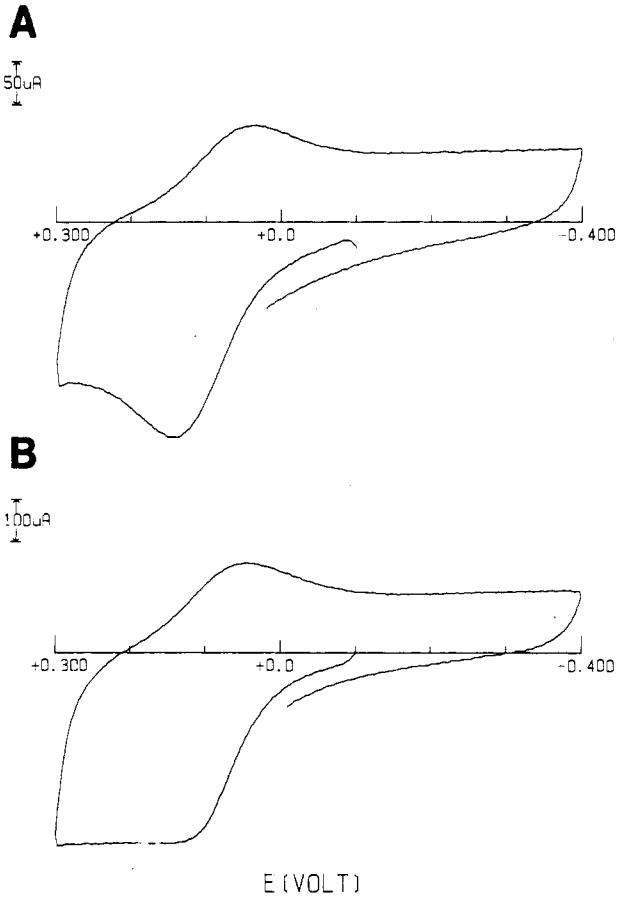
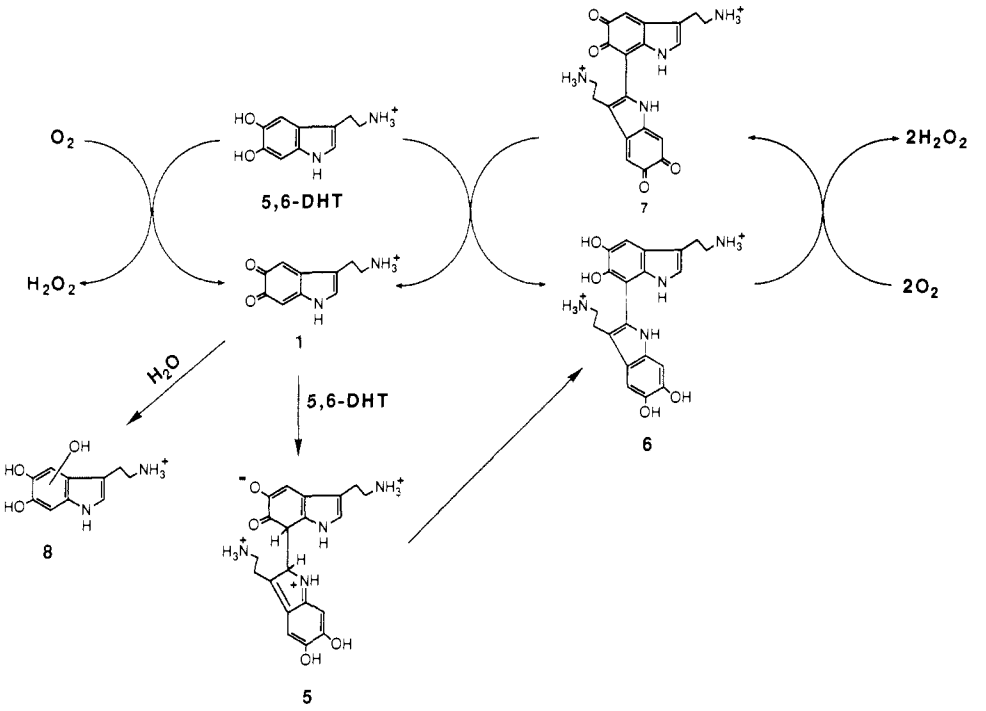


Figure 5. Cyclic voltammograms at a platinum electrode of (A) 0.5 mM 5,6-DHT and (B) 0.5 mM **6** obtained in pH 7.2 phosphate buffer (μ = 0.1). Sweep rate was 20.48 V s^{-1}.

Electrochemical Studies. A representative cyclic voltammogram of 5,6-DHT obtained at a platinum electrode at pH 7.2 is shown in Figure 5A. On the first anodic sweep, an oxidation peak appears and, after scan reversal, a quasireversible reduction peak appears. At sweep rates <2 V s^{-1} the reverse reduction peak decreases in height and at <0.5 V s^{-1} it disappears. These behaviors indicate that the initial oxidation product of 5,6-DHT undergoes a relatively rapid chemical reaction and hence disappears at slow sweep rates. At sweep rates ≥2 V s^{-1} the experimental peak current function, $i_p/AC\nu^{1/2}$ (where i_p is the peak current, A the electrode area, C concentration of

Scheme II



5,6-DHT, and ν the voltage sweep rate), for the oxidation peak of 5,6-DHT was independent of ν. Similarly, i_p linearly increased with the concentration of 5,6-DHT (0.1–1 mM). Such results indicate that the voltammetric oxidation is under linear diffusion control. Assuming a reasonable value for the diffusion coefficient of 5,6-DHT, 5×10^{-6} cm^2 s^{-1},[20] i_p for the oxidation peak was employed to calculate the number of electrons, n, transferred per molecule in the electrode process,[21] assuming that this process is electrochemically reversible. At $\nu \geq 2$ V s^{-1} the calculated n value was 2 ± 0.05. Between pH 4 and 8 the peak potential (E_p) for the voltammetric oxidation peak of 5,6-DHT shifted with pH according to the relationship $\partial E_p/\partial pH = -59$ mV. Hence, it may be concluded that 5,6-DHT is electrooxidized in a 2e,2H$^+$ reaction to *o*-quinone 1. The reverse reduction peak observed in the cyclic voltammogram of 5,6-DHT corresponds to the 2e,2H$^+$ reduction of 1 to 5,6-DHT. The formal potential, $E^{0\prime}$, for the 5,6-DHT/1 couple computed from eq 1 was

$$E^{0\prime} = \frac{E_{p_{ox}} + E_{p_{red}}}{2} \quad (1)$$

0.088 V. Controlled-potential electrooxidation of 5,6-DHT (0.5 mM) at 0.08 V for periods ranging from 30 min to 4 h 15 min in pH 7.2 phosphate buffer gave dimer **6** as the major product. The latter was confirmed by HPLC analysis, UV spectra, and cyclic voltammetry of the isolated product.

A cyclic voltammogram of **6** at pH 7.2 (Figure 5B) showed an almost reversible couple at similar potentials ($E^{0\prime} = 0.099$ V) to that observed with 5,6-DHT. At sweep rates ≥ 5 V s^{-1}, at which the electrochemistry of both 5,6-DHT and **6** are not complicated by follow-up chemical reactions, i_p for **6** was approximately twice that for 5,6-DHT. Since the diffusion coefficient of **6** must be somewhat smaller than that of 5,6-DHT, it may be concluded that the electrochemical oxidation of **6** is a 4e reaction.[20]

Discussion

Autoxidation of 5,6-DHT at physiological pH is a complex process. Spectral changes (Figure 1) indicate that the autoxidation exhibits features characteristic of an autocatalytic reaction, an effect noted in previous investigations.[4] The inhibitory effects of catalase, SOD, and DTPA indicate that H_2O_2, $O_2^{\bullet-}$, and trace contamination by transition metal ions, respectively, play active roles in the overall autoxidation reaction. However, when transition metal ions are complexed with DTPA and O_2 is excluded, 5,6-DHT and its major initial oxidation product **6** are not significantly oxidized by H_2O_2. Thus, H_2O_2 does not directly oxidize 5,6-DHT as proposed by Klemm et al.[4] Additions of very low concentrations of Fe^{3+} or Cu^{2+} to the reaction mixture results in a significant enhancement in the rate of the autoxidation, indicating that such ions exert a catalytic effect. In situ generation of $O_2^{\bullet-}$ also results in an acceleration of the autoxidation process.

Cyclic voltammetry indicates that 5,6-DHT is oxidized in a 2e,2H$^+$ reaction to give quinone 1 and that the redox potential, $E^{0\prime}$, for the 5,6-DHT/1 couple is +0.088 V (+0.328 V vs NHE). The value of $E^{0\prime}$ for the $O_2/O_2^{\bullet-}$ couple in aqueous solution at pH 7.2, determined on the basis of pulse-radiolysis experiments, is –0.33 V vs NHE.[22] Thus, it is highly improbable that the initial step in the autoxidation reaction is a direct one-electron transfer from 5,6-DHT to O_2. The $E^{0\prime}$ for the O_2/H_2O couple at pH 7 is +0.815 V[23] and, at least in principle could account for the oxidation of 5,6-DHT. However, this reaction cannot account for the production of H_2O_2 and $O_2^{\bullet-}$, which are clearly formed in the autoxidation reaction, or for the catalytic influence of transition metal ions such as Fe^{3+} or Cu^{2+}. Dioxygen can also undergo a two-electron reduction to H_2O_2. The value of $E^{0\prime}$ for the O_2/H_2O_2 couple in aqueous solution at pH 7 is 0.36 V vs NHE.[24] Ac-

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cordingly, we propose that the initial step in the autoxidation of 5,6-DHT involves the transfer of two electrons and two protons to O_2 with concomitant formation of quinone 1 and H_2O_2. Nucleophilic attack by 5,6-DHT on 1 leads to the 2,7′-linked dimer **6** as conceptualized in Scheme II.

Autoxidation of dimer **6** is a significantly faster reaction than is autoxidation of 5,6-DHT and hence **6** is rapidly oxidized. It has not been possible to isolate and characterize the initial or, indeed, any oxidation product of **6** because of rapid polymerization reactions. However, electrochemical experiments suggest that **6** is oxidized in a 4e,4H$^+$ reaction. Thus, it seems reasonable to conclude that **6** is oxidized to diquinone **7** along with concomitant formation of H_2O_2 (Scheme II). Although **6** is more rapidly autoxidized than 5,6-DHT, it nonetheless accumulates in solution during the early stages of the autoxidation reaction. This implies that a mechanism exists whereby **7** is reduced to **6**. The $E^{0\prime}$ values for the **6**/**7** couple is slightly more positive than that for the 5,6-DHT/1 couple. Accordingly, it is proposed that **7** chemically oxidizes 5,6-DHT to 1. This reaction is more likely in the early stages of the autoxidation reaction when there is a large excess of the reducing agent 5,6-DHT. The reactions shown in Scheme II indicate that in principle 1 mol of 5,6-DHT is oxidized to 1 mol of dimer **6** with concomitant formation of 1 mol of H_2O_2. Autoxidation of **6** to **7** then generates 2 additional mol of H_2O_2. Each mole of **7** can then oxidize 2 mol of 5,6-DHT to 1 and, thence, 2 mol of **6** and then **7** are formed. Such a series of reactions represents an autocatalytic cycle. Two trihydroxyindolamines are known to be very minor products of autoxidation of 5,6-DHT and are represented as **8** in Scheme II.[12] These are probably formed as a result of nucleophilic attack by water on *o*-quinone 1.

Autoxidations of 5,6-DHT and **6** generate H_2O_2 as a byproduct. In the presence of transition metal ions H_2O_2 clearly plays an important role in the overall autoxidation chemistry of both 5,6-DHT and **6**. However, as noted previously, H_2O_2 in the absence of trace transition metal ions cannot oxidize either compound. Thus, it may be concluded that trace transition metal ions interact with H_2O_2 to effect oxidation of 5,6-DHT and **6**. This conclusion is further supported by the considerable increase in the rate of the autoxidation reaction caused by addition of micromolar concentrations of Fe^{3+} and Cu^{2+}. Transition metal ions such as Fe^{2+},[25,26] Cu^+,[27] and Co^{2+}[28] can catalyze the reduction of H_2O_2 to generate hydroxyl radical via the well-known Fenton reaction. However, this reaction necessarily requires reduction of the higher oxidation state of the metal ions expected under normal conditions. Because of its ubiquitous nature, the following discussion will focus on reactions of iron ions. It is well-known that Fe^{3+} can catalyze the decomposition of H_2O_2 according to eq 2, generating the hydroperoxy radical, $HO_2^\bullet$, and Fe^{2+}.[26,29]

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^+ + HO_2^\bullet \quad (2)$$

This reaction has been studied most extensively in dilute acid solution because of the complicating effects of the oxides of iron on the kinetics of the reaction at higher pH values. However, similar reactions occur in neutral or alkaline aqueous solutions of H_2O_2.[30,31] Accordingly, reaction 2 can serve as a source of Fe^{2+}. It is also worth noting that Cu^{2+} can also catalyze reaction 2, and furthermore, Cu^{2+} is directly reduced by 5,6-DHT. The reduced form of copper so generated could also drive Fenton chemistry directly or indirectly by reduction of Fe^{3+} to Fe^{2+}. The Fenton reaction (eq 3) leads to formation of hydroxyl radical, $HO^\bullet$. This radical then reacts directly

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^\bullet \quad (3)$$

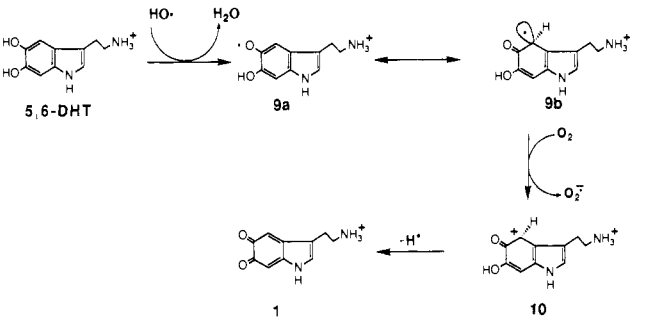
$$HO^\bullet + H_2O_2 \rightarrow H_2O + HO_2^\bullet \qquad k = 3 \times 10^7\ M^{-1}\ s^{-1} \quad (4)$$

$$HO_2^\bullet \rightleftharpoons H^+ + O_2^{\bullet-} \qquad pk_a = 4.8 \quad (5)$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (6)$$

with H_2O_2 to generate $HO_2^\bullet$ (eq 4) which at pH 7.2 dissociates to give $O_2^{\bullet-}$ (eq 5).[32] In aqueous solution $O_2^{\bullet-}$ is a moderately strong reducing agent[33] and hence reduces Fe^{3+} to Fe^{2+} (eq 6). Reaction 2 also serves as a second source of $HO_2^\bullet$ and hence $O_2^{\bullet-}$ to drive reaction 6. It is known from results reported earlier that H_2O_2 cannot directly oxidize 5,6-DHT or **6**. Futhermore, $O_2^{\bullet-}$ is known to be a pitifully weak oxidizing agent (roughly the equivalent of Na^+)[33] and could not possibly directly oxidize 5,6-DHT or **6**. Accordingly, it must be concluded that the reaction sequence described in eqs 2–6 must serve as a route to generate $HO^\bullet$. The hydroxyl radical is a very powerful one-electron oxidizing agent[23] and hence we propose that it oxidizes 5,6-DHT to the intermediary radical **9a**/**9b** (Scheme III). Such a radical can be expected to be as or more easily oxidizable than 5,6-DHT.[34] Hence it is not unreasonable to suggest that **9a**/**9b** transfers an electron to O_2, forming $O_2^{\bullet-}$ and quinone **1** as outlined in Scheme III. The latter species would then undergo the follow-up reactions described in Scheme II. At the stage in the reaction where appreciable quantities of **6** accumulate, it is probable that $HO^\bullet$ reacts with the dimer in a reaction similar to that outlined for 5,6-DHT in Scheme III. Reactions of the sort described in the latter scheme generate $O_2^{\bullet-}$, which would serve to reduce Fe^{3+} to Fe^{2+} (eq 6) and hence drive the Fenton reaction (eq 3) and generate $HO^\bullet$. Support for the pathway outlined in Scheme III derives from two sources. First, in the presence

Scheme III



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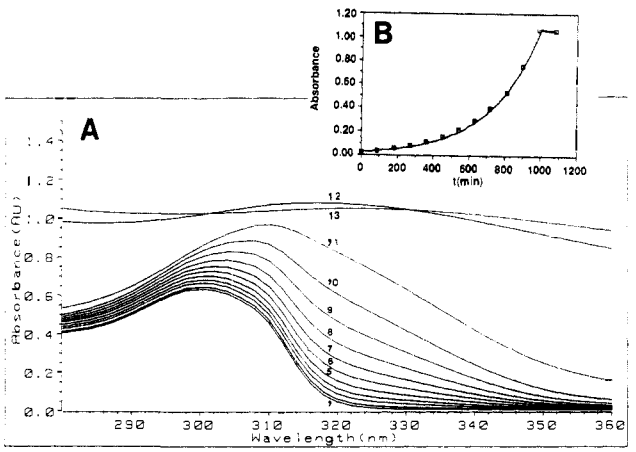

Figure 6. (A) Spectral traces recorded during the autoxidation of 0.2 mM 5,6-DHT (0.16 mg in 2 mL of pH 7.2 phosphate buffer, μ = 0.1) in the presence of DTPA (0.77 mg, 1 mM), catalase (1 mg, 2000 units), and SOD (50 μL, 1000 units). Curve 1 is the spectrum of 5,6-DHT; curves 2–13 were recorded at 90-min intervals. (B) Absorbance at 324 nm versus time for the reaction described in A. The experiment was carried out at room temperature.

of peroxidase enzymes the rate of O_2 consumption increases appreciably above that observed in the autoxidation reaction. Peroxidase usually acts as a one-electron oxidant of substrates to yield radical intermediates.[35,36] Thus, it is likely that peroxidase/H_2O_2 generates radical intermediate **9a**/**9b**, which reacts directly with O_2 to generate quinone 1 and $O_2^{\bullet-}$ as described in Scheme III. Such a process would account for the enhanced rate of O_2 consumption noted in the presence of peroxidase. Secondly, addition of H_2O_2 to the autoxidation reaction system both in the absence and presence of peroxidase increases the rate of oxygen consumption. In both of these situations the rate of formation of radical **9a**/**9b** should be enhanced. In the absence of peroxidase this would be effected by reactions 2–6; i.e., the rate of HO$^\bullet$ formation should be increased.

Scheme II predicts that autoxidation of 5,6-DHT should exhibit autocatalytic behavior even in the absence of reactants deriving from H_2O_2 (i.e., HO$^\bullet$, $O_2^{\bullet-}$). This prediction was investigated by studying the autoxidation of 5,6-DHT in the presence of DTPA, catalase, and SOD. These agents should inhibit the influences of H_2O_2, Fe^{3+} (or other transition metal ions), and $O_2^{\bullet-}$ on the autoxidation reaction. Spectra recorded throughout the autoxidation are presented in Figure 6A. Very little reaction occurs during the initial 3–4 h, but with time there is a clear acceleration in the reaction. This is most readily observed from Figure 6B, which shows a plot of the absorbance at 324 nm versus time. This clearly indicates that in the absence of reactions deriving from H_2O_2, $O_2^{\bullet-}$, and Fe^{3+} the autoxidation of 5,6-DHT is autocatalytic. The spectral changes observed in Figure 6A reveal that over the course of 18 h 5,6-DHT is converted to dimer **6** and that very little polymeric precipitate is formed. Only during the final very rapid stages of the reaction, which must correspond largely to autoxidation of **6**, does black melanin pigment form with the resulting rapid increase in absorbance over the entire spectral region. The slow rate of autoxidation of 5,6-DHT under the latter conditions was confirmed by oxygen electrode measurements. The initial oxygen consumption rate was 1.48 nmol of O_2/min at 37 °C.

Tyrosinase and ceruloplasmin clearly catalyze the oxidation of 5,6-DHT to dimer **6** and thence to a polymeric indolic melanin. The mechanisms of these enzyme-mediated oxidations were beyond the scope of this work. Nevertheless, the mechanism proposed by Hamilton[37] for tyrosinase when it functions as an oxidase would predict formation of *o*-quinone 1. The ceruloplasmin-mediated oxidation of 5,6-DHT most probably proceeds via an initial one-electron oxidation to a radical intermediate (**9b**/**9b**) which could either be further oxidized (1e) by ceruloplasmin to quinone 1 or be attacked by O_2 as conceptualized in Scheme III.

Mitochondria also promote the autoxidation of 5,6-DHT to dimer **6** and thence to melanin polymer. The enhanced oxidation rate is not blocked by monoamine oxidase (MAO) inhibitors, which indicates that there is no interaction with mitochondrial MAO.[4,5] Cohen and Heikilla[5] have proposed that 5,6-DHT can act as an electron donor in the electron-transport chain in mitochondria to form a quinone oxidation product. The results reported here are not in disagreement with the latter suggestion.

Conclusions

The results presented above indicate that autoxidation of the serotonergic neurotoxin 5,6-DHT is initiated by molecular oxygen, leading to formation of quinone 1 and H_2O_2. Quinone 1 is attacked by 5,6-DHT, yielding dimer **6**. This dimer is more rapidly oxidized by O_2 than is 5,6-DHT to give diquinone **7** and 2 mol of H_2O_2. Thus, one oxidative cycle from 5,6-DHT to **7** liberates 3 mol of H_2O_2. Diquinone **7** can itself oxidize 2 mol of 5,6-DHT to 2 mol of **6**, which are oxidized by O_2 to yield 2 mol of H_2O_2. Thus an autocatalytic cycle is established. In the absence of any influences from decomposition of H_2O_2, this autocatalytic reaction is represented kinetically by the data presented in Figure 6B. However, in the absence of agents which protect the system from the latter effects, the H_2O_2 generated can interact with trace transition metal ions such as Fe^{3+} and Cu^{2+} with resultant formation of a second catalytic cycle which generates HO$^\bullet$. The latter radical can oxidize 5,6-DHT to a radical intermediate (**9a**/**9b**) which consumes O_2 to form quinone 1 and $O_2^{\bullet-}$. The former can continue through the cycle represented in Scheme II while the latter can reduce Fe^{3+} to Fe^{2+}, which can further generate HO$^\bullet$ via the Fenton reaction (eq 3). As the autoxidation progresses polymeric melanin is generated as a result of further reactions between **7** and, presumably, **6**, 5,6-DHT, and **8**, although it has not yet been possible to investigate the course of this chemistry. Experiments with tyrosinase, ceruloplasmin, and rat brain mitochondria indicate that oxidative systems endogenous to the central nervous system may catalyze the oxidation of 5,6-DHT. While it has not yet been possible to assess whether the chemistry elucidated in vitro occurs in the CNS, the information provided in this and earlier reports indicate that such chemistry is likely. Reactions of quinone 1 or indeed quinone **7** with endogenous nucleophiles remain to be studied. However, the facile reaction between quinone 1 and 5,6-DHT suggests that such reactions are probable. The very rapid polymerization of **7** also suggests that this electrophilic diquinone might represent an important target for endogenous nucleophiles and hence provide additional routes for alkylation reactions proposed to explain the neurodegenerative properties of 5,6-DHT of the

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type outlined in Scheme I. An alternative route to explain the neurodegenerative properties of 5,6-DHT might be related to HO•, which is almost certainly formed as a result of the autoxidation of 5,6-DHT, 6, and perhaps larger oligomeric indolamines which must serve as precursors of the ultimate high molecular weight melanin product. The generation of HO• relies upon the decomposition of H_2O_2 by Fenton reactions which necessarily require trace concentrations of transition metal ions. Although Cu^+ and Co^{2+} are capable of serving in Fenton chemistry, the preponderance of cellular iron suggests that it is the most probable catalyst for the Fenton reaction in vivo. Furthermore, it is not necessary that the transition metal ion catalyst be in its uncomplexed (solvated) form since there are many reports that complexed or bound iron can participate in Fenton chemistry.[38,39]

It is also of interest to consider the potential role of antioxidants endogenous to the CNS in the oxidation chemistry of 5,6-DHT. Glutathione (GSH), for example, is thought to occur in the CNS at about the 1–2 mM concentration level.[40] Preliminary experiments (data not reported) indicate that 5,6-DHT in the presence of O_2 mediates the oxidation of GSH to the corresponding disulfide (GSSG). Thus, autoxidation of 5,6-DHT generates 1 with concomitant formation of H_2O_2. Quinone 1 then oxidizes GSH to GSSG. In the presence of mitochondria, or perhaps many other endogenous oxidative enzymes, this reaction might be greatly accelerated. Hydrogen peroxide is eliminated in brain tissue principally by glutathione peroxidase.[41] Thus, the H_2O_2 generated as a byproduct of autoxidation of 5,6-DHT should result in further conversion of GSH to GSSG. As a result of this redox cycling of the 5,6-DHT/1 system, the endogenous concentrations of GSH in the vicinity of 5,6-DHT should be drastically diminished. Under these conditions further autoxidation of 5,6-DHT with concomitant formation of H_2O_2 should permit Fenton chemistry and hence HO• production to assume a major neurodegenerative role.

Oxidation chemistry and biochemistry of 5,6-DHT can lead to the formation of electrophilic quinone intermediates such as 1 and 7, which might cause neuronal degeneration as a result of the alkylation of membrane proteins. Redox cycling reactions might result in substantial decreases in endogenous but localized concentrations of antioxidants such as glutathione and to hypoxic states within the neuron (a state which causes a cell to become highly susceptible to injury by reduced oxygen species[42]). Reactions of H_2O_2 generated as a byproduct of the autoxidation of 5,6-DHT might serve as a source of HO•, which could inflict neuronal injury. However, at this stage of our investigations it is not yet possible to establish whether all or any of these processes are responsible for the neurodegenerative properties of 5,6-DHT.

Experimental Section

5,6-Dihydroxytryptamine (creatinine sulfate salt), tyrosinase (mushroom, EC 1.14.18.1), peroxidase (types VI, VIII, IX, and X from horseradish, EC 1.11.1.7), xanthine oxidase (from milk in 2.3 M ammonium sulfate containing 0.02% sodium salicylate, EC 1.1.3.22), superoxide dismutase (bovine, suspension in 3.8 M ammonium sulfate solution pH 7.0, EC 1.15.1.1), ceruloplasmin (human, type X in pH 7.0 0.25 M sodium chloride/0.05 M sodium acetate), and catalase (bovine liver, EC 1.11.1.6) were obtained from Sigma (St. Louis, MO) and were used without further purification.

Phosphate buffers of known ionic strength (μ) at pH 7.2 were prepared according to Christian and Purdy.[43] 3-*N*-Morpholinopropane sulfonic acid (MOPS, sodium salt) and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, sodium salt) were also used to prepare buffers at pH 7.2 (μ = 0.1).

Rat brain mitochondria were freshly prepared accordingly to the modified procedure of Clark and Nicklas[44] and were assayed by using conventional procedures.[45]

High-performance liquid chromatography (HPLC) employed a Bio-Rad gradient instrument equipped with dual pumps, and Apple Model II_e controller, a Rheodyne Model 7125 loop injector, and an Isco Model 226 UV detector (254 nm). A reversed-phase column (Brownlee Laboratories, RP-18, 5-μm particle size, 250 × 7 mm) was employed which was protected by a short guard column (Brownlee, RP-18, 5 μm, OD-GU, 50 × 5 mm). The mobile-phase solvents were prepared as follows: Solvent A was prepared by adding 20 mL of HPLC-grade methanol (Fisher Scientific) and 10 mL of 28–30% ammonium hydroxide to 970 mL of deionized water. The pH of this solution was adjusted to 3.25 with concentrated formic acid. Solvent B was prepared by adding 400 mL of HPLC-grade methanol and 10 mL of 28–30% ammonium hydroxide to 590 mL of deionized water. The pH of this solution was then adjusted to 3.25 with concentrated formic acid. The gradient employed was as follows: 0–16 min, 100% solvent A at a flow rate of 1.5 mL min^{-1}; 16–35 min, linear gradient to 5% solvent B and a corresponding increase of flow rate to 2.5 mL min^{-1}; 35–50 min, linear gradient to 60% solvent B and a linear increase of flow rate to 3.0 mL min^{-1}. The latter mobile-phase composition was then maintained for 6 min with a linear increase of flow rate to 3.5 mL min^{-1}. The mobile phase was then linearly returned to 100% solvent A over 4 min. The column was equilibrated with 100% solvent A for 10 min (1.5 mL min^{-1}) before another sample was injected. Typically, a 2.0-mL sample volume was injected into the HPLC system.

UV–visible spectra were recorded on either a Hitachi 100-80 spectrophotometer or a Hewlett-Packard 8452A diode-array spectrometer.

Cyclic voltammetric studies employed a Bioanalytical Systems (BAS, West Lafayette, IN) Model 100A electrochemistry system. All voltammograms were obtained at a platinum disc working electrode and were corrected for iR drop. The area of the platinum electrode (0.025 cm^2) was measured by using a potentiostatic method with 4.0 mM potassium ferrocyanide in 0.5 M potassium chloride as described by Adams.[20] A conventional electrochemical cell containing a platinum wire counter electrode and saturated calomel reference electrode (SCE) was employed. Unless otherwise noted, all potentials are referred to the SCE at ambient temperature (22 ± 2 °C). All test solutions were thoroughly deaerated with a vigorous stream of nitrogen for about 3 min before voltammograms were recorded. Conventional equipment was employed for controlled potential electrolyses. However, the working electrode consisted of several plates of pyrolytic graphite (Pfizer Minerals, Pigments and Metals Division, Easton, PA). Solutions undergoing controlled potential electrolyses were continuously stirred and nitrogen was vigorously bubbled through the solution.

Oxygen consumption was measured with a Clark-type oxygen electrode assembly (YSI, Model 5300). In order to monitor oxygen consumption 3.0 mL of 1.0 mM 5,6-DHT in pH 7.2 buffer solution was incubated at 37 °C. In some instances an aliquot, typically 1.0 mL, of this solution was removed after an appropriate time and analyzed by HPLC or spectrophotometry. However, in order to more completely monitor various oxidation reactions, larger volumes of solution were employed. In some instances these solutions were exposed to the atmosphere while being thermostated at 37 °C. In other cases nitrogen gas was bubbled vigorously through the solution. Typically, 2.0-mL aliquots of these solutions were periodically removed and injected into the HPLC system.

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Spectral changes which accompanied the oxidation employed 0.20 mM 5,6-DHT in pH 7.2 buffer at room temperature in a 0.5-cm quartz cell.

Unless otherwise noted, the pH 7.2 phosphate buffer employed had an ionic strength (μ) of 0.1.

Acknowledgment. The support of this work through grants from the National Institutes of Health through Grant No. GM-32367, the Oklahoma Center for the Advancement of Science and Technology through Contract No. 3745 HR8-072, and by the Research Council of the University of Oklahoma is gratefully acknowledged. We also thank Dr. R. N. Goyal for some of the electrochemical experiments which were performed.

Registry No. 1, 129176-09-6; 5, 129176-12-1; 6, 129176-08-5; 7, 129176-10-9; 8 (isomer 1), 129176-11-0; 8 (isomer 2), 55206-15-0; 9a, 129176-13-2; 5,6-DHT, 5090-36-8; H_2O_2, 7722-84-1; SOD, 9054-89-1; Fe, 7439-89-6; Cu, 7440-50-8; OH, 3352-57-6; $O_2^{\cdot-}$, 11062-77-4; creatinine, 60-27-5; catalase, 9001-05-2; peroxidase, 9003-99-0; xanthine oxidase, 9002-17-9; tyrosinase, 9002-10-2; xanthine, 68-89-6; ceruloplasmin, 9031-37-2.

Potential Antimitotic Agents. Synthesis of Some Ethyl Benzopyrazin-7-ylcarbamates, Ethyl Pyrido[3,4-*b*]pyrazin-7-ylcarbamates, and Ethyl Pyrido[3,4-*e*]-*as*-triazin-7-ylcarbamates

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Ring analogues and derivatives of the 1,2-dihydropyrido[3,4-*b*]pyrazin-7-ylcarbamates (e.g., **29**), antimitotic agents with antitumor activity, were prepared in the search for compounds with greater selectivity. Methods were developed for the conversion of substituted benzoic acids (**1–4**) to give benzopyrazines (**12–16** and **21**) and of substituted pyridin-2-carbamates (**23**, **38**, and **41**) to give 2-aminopyrido[3,4-*b*]pyrazin-7-ylcarbamates (**32** and **36**) and pyrido[3,4-*e*]-*as*-triazin-7-ylcarbamates (**47** and **50**). In vitro evaluation indicated that activity was reduced by removal of the pyridine ring nitrogen of **29** to give **14** and was destroyed by increasing the basicity of the pyrazine ring of **29** to give **32** and **47**.

The 1,2-dihydropyrido[3,4-*b*]pyrazin-7-ylcarbamates (e.g., **29**) are potent inhibitors of the in vitro polymerization of tubulin to give microtubules.[1] These compounds show anticancer activity and are cytotoxic to cultured L1210 cells at nanomolar concentrations.[2,3] The preparation of compounds with greater cytotoxicity, however, has not provided compounds with greater antitumor activity. In work directed toward the identification of agents with greater selectivity, ring analogues and derivatives of the pyrido[3,4-*b*]pyrazines were synthesized. The effect on activity of removing the pyridine ring nitrogen was determined by the preparation of benzopyrazines, and the effect on activity of increasing the basicity of the pyrazine ring was determined by the preparation of pyrido[3,4-*e*]-*as*-triazines and 2-aminopyrido[3,4-*b*]pyrazines.

Chemistry

The commercially available 4-chloro-3,5-dinitrobenzoic acid (**1**) was converted to the corresponding acid chloride (**2**), ethyl ester (**3**), and acid azide (**4**) by the reported methods.[4] The amination of **3** with the diethyl acetal of 2-amino-2-phenylacetaldehyde[5] gave **6**, which was hydrogenated over Raney nickel to give **9** (Scheme I). Without isolation **9** was treated with acid, and the cyclization product was allowed to undergo air oxidation to afford **12**. Reduction of **12** with $NaBH_4$–Al_2O_3 gave **13**, which was isolated as a 4:1 mixture of **13** and **12**. A similar route was attempted for the preparation of **14**. The isocyanate formed from **4** in hot toluene was reacted with methanol to give **5**. Also the preparation of **5** was attempted from **2** and excess sodium azide. Under these conditions, the ring chloro group was displaced by an azido group, which underwent loss of N_2 with formation of **17**.[6] Amination of **5** with the diethyl acetal of 2-amino-2-phenylacetaldehyde[5] gave **7**, which was hydrogenated over Raney nickel in ethanol to give crude **10**. The presence of **10** in this sample was supported by the mass spectrum and the absence in the ^1H NMR spectrum of **14** (or tautomer) and its oxidization product **15**. Cyclization of **10** to **14** was attempted with acid, however, these conditions resulted in the loss of the acetal moiety with the formation of the benzimidazole **20**. This reaction probably proceeds by the addition of either of the adjacent amino groups to the α-carbon of the enol form of the aldehyde followed by loss of formaldehyde and oxidation of the resulting dihydrobenzimidazole. The structure of **20** was confirmed by an unambiguous synthesis. The amination of **5** with ammonia gave **18**, which was hydrogenated over Raney nickel to give **19**. The oxidative cyclization of **19** with the sodium bisulfite addition product of benzaldehyde gave **20**, a reaction that also proceeds via a dihydrobenzimidazole intermediate.[7]

In another approach to **14**, **5** was reacted with methyl 2-phenylglycinate[8] to give **8**. Hydrogenation of **8** over Raney nickel gave **11**, which was cyclized to give a mixture of **21** (86%) and its oxidized product **16** (14%). Also, the reductive cyclization of **8** was effected with Zn–HOAc, but in this reaction the product (**21**) was allowed to undergo air oxidation to give **16**. Reduction of the amide group of

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0022-2623/90/1833-3044$02.50/0